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We are using an intrabody approach to phenotypically knock out cyclin E expression in breast cancer cells. Cyclin E is overexpressed in almost all breast cancer cell lines and patient tumor tissue examined to date. However, the role cyclin E plays in breast cancer tumorigenesis and proliferation is yet to be elucidated. We have generated two anti-cyclin E single-chain antibodies (sFvs) and have generated mammalian vectors for their expression as Fc fusion proteins to assess their ability to bind in vitro to cyclin E. We have also generated mammalian expression vectors to target sFv or sFv-Fc expression to the cytosol or nucleus of breast cancer cells. We have displayed the ability to successfully target sFv and sFv-Fc to the cytosol and nucleus of MCF-7 and SKBR-3 cells, and are currently developing cell line(s) to evaluate the biological effects of anti-cyclin E intrabodies on breast cancer cells.

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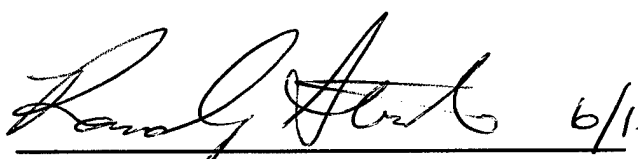

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Table of Contents

Cover.....

SF 298.....

Foreword.....

Introduction..... 4

Body..... 4

Key Research Accomplishments..... 6

Reportable Outcomes..... 6

Conclusions..... 6

References.....

Appendices..... 7

Introduction

Cyclin E is an important regulator of the coordinated cell cycle, as it is rate limiting for the G1/S phase transition. Deranged cyclin E expression has been found both quantitatively (overexpression) and qualitatively (multiple isoforms) in almost all breast cancer cell lines and patient tumor tissue examined to date. Disruption of such an important cell cycle protein could potentially contribute to transformation and/or tumorigenicity in breast cancer, but the direct involvement of cyclin E in breast cancer has yet to be elucidated. Intracellular antibodies, or intrabodies, are a powerful tool for evaluating the *in vivo* function of a given protein. Single-chain antibodies (sFvs), consisting of the heavy and light chain variable regions of an IgG linked by a flexible peptide spacer, are able to fold and retain the antigen binding specificity of the parental antibody. SFvs can be modified for targeted expression to the cytosol, nucleus, or endoplasmic reticulum (ER), where they can bind to and inactivate their target antigen, thus generating a "phenotypic knockout" of the protein of interest. In this study we have constructed two anti-cyclin E sFvs and generated constructs that successfully target their expression as intrabodies to the cytosol or nucleus of breast cancer cells. We propose to use breast cancer cell lines stably or inducibly expressing these anti-cyclin E intrabodies to evaluate their effect on the nuclear trafficking and associated kinase activity of cyclin E. We will then evaluate the biological effects (i.e. doubling time, cell cycle distribution, growth in soft agar, tumorigenicity in nude mice) of anti-cyclin E intrabody expression in these cell lines. This study should provide direct evidence to determine the functional significance of cyclin E in the abnormal growth and transformation of breast cancer, as well as determine if there is merit in cyclin E targeted strategies for the treatment of breast cancer.

Annual Summary

The first aim of this proposal was to generate anti-cyclin E single chain antibodies (sFvs) based on the parental anti-cyclin E monoclonal antibody producing hybridoma cell lines HE-12 and HE-172. These hybridomas were generated and characterized in the lab of Dr. Edward Harlow at Harvard University. We obtained these hybridomas and grew them in culture for the purpose of isolating poly-adenylated messenger RNA (mRNA) from log phase cells using the Micro-FastTrack mRNA Isolation Kit (Invitrogen). Using this mRNA as template, we then performed RT-PCR to amplify the approximately 350 base pair cDNA fragments which encode the heavy (V_H) and light (V_K) chain variable regions of the anti-cyclin E antibodies (Fig 1). RT-PCR was performed using degenerate primers designed against the Framework 1 (5') and Framework 4 (3') regions of the IgG heavy and light chain genes. These primers incorporated HindIII (5') and XbaI (3') restriction sites to facilitate cloning into a sequencing vector. Several clones were sequenced and the resulting nucleotide and deduced amino acid sequences were subjected to GeneBank query to ensure they encoded for unique peptide sequences. Unique heavy and light chain cDNAs were PCR amplified to add overlapping oligonucleotides encoding a $(Gly_4Ser)_3$ linker, and the two fragments were then linked by an additional overlap extension PCR into sFvs in the order of V_H-Linker-V_K (Fig 1).

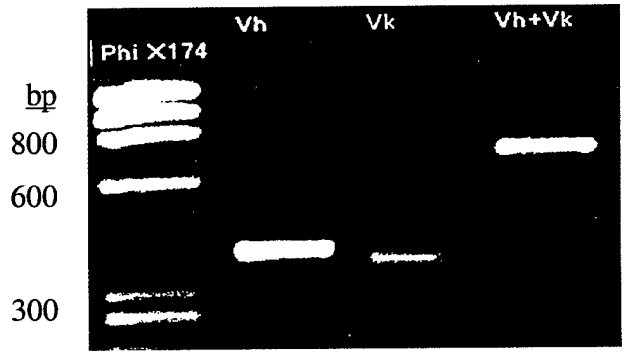


Figure 1

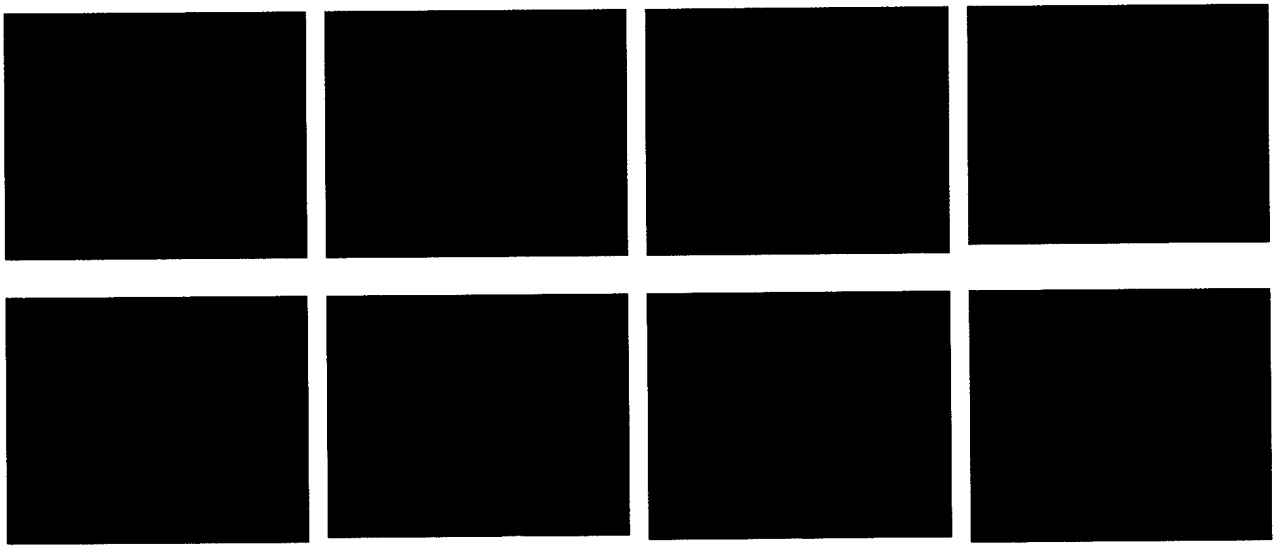
Using this procedure, we have successfully cloned the heavy and light chain variable region genes of both the HE-12 and HE-172 hybridomas, and linked them into sFvs. The nucleotide and deduced amino acid sequences for these sFv constructs are depicted in Appendix A. The first construct to be completed was that from the HE-172 cell line, and we initially cloned the HE-172 construct into a baculovirus expression system for expression and purification of sFvs in Sf9 insect cells, and assessment of cyclin E binding affinity by Western blot or ELISA. Although we have successfully expressed epitope tagged (influenza Hemaglutanin Antigen-*HA*) HE-172 sFv in insect cells (Figure 2), purification of sufficient protein for binding analysis has not been achieved using the baculovirus system. We have recently completed cloning of both intrabody constructs into a mammalian expression vector (pRc/CMV) with the goal of producing

secreted sFv-Fc fusion proteins by cloning the sFv constructs in frame with the cDNA encoding the human IgG Fc fragment. Addition of the Fc fragment has been shown to increase protein stability, enhances sFv secretion, and facilitates purification of the sFv-Fc fusion proteins by Protein A affinity chromatography. The IgG heavy chain signal sequence was added 3' to the sFv-Fc constructs to target them to the secretory pathway. These constructs are currently being evaluated in COS-1 cells.

Figure 2. Expression of HE-172 sFv-HA in Sf9 insect cells. **A.** Sf9 cells infected with recombinant baculovirus carying the HE-172 sFv-HA construct (left) or mock infected cells (right) were fixed and stained with anti-HA FITC antibody 48 hrs post-transfection. **B.** Supernatants from infected or mock infected Sf9 cells were separated by SDS-PAGE, transferred to nitrocellulose, blocked, blotted with rabbit anti-HA (1°), and anti-Rabbit-HRP (2°) antibodies, and developed using ECL detection kit (Amersham).

The second aim of this proposal was to generate mammalian expression vectors for use in the targeted expression of anti-cyclin E intrabodies to the cytosol or nucleus of the breast cancer cell lines MCF-7 and SKBR-3. A schematic representation of the constructs for cytosolic and nuclear expression of sFv and sFv-Fc intrabodies is shown in Appendix B. All of the constructs depicted have been completed for the HE-172 sFv, and were used in transient transfection assays using MCF-7 and SKBR-3 cells. The sFv was modified by addition of the HA epitope tag for use in immunodetection. Additional sFv-Fc constructs were made to assess the effect of the Fc fragment on expression, stability, and targeting of sFvs in breast cancer cells. Antibodies specific for the human Fc fragment were used for immunodetection of sFv-Fc constructs. Addition of the SV40 nuclear localization signal (NLS) to the 3' end of the sFv or sFv-Fc construct was used to target expression of the protein to the nuclear compartment. Figure 3 presents immunofluorescence results from transient transfection of MCF-7 and SKBR-3 cells. Briefly, MCF-7 or SKBR-3 cells were grown in 6-well plates and transfected with the indicated constructs using GenePorter transfection reagent (Gene Therapy Systems). 48 hours post-transfection, cells were fixed and stained with either anti-HA-FITC or anti-human Fc-FITC antibody. These results indicated the ability to successfully target expression of sFv and sFv-Fc intrabodies to the cytosol or nucleus of both MCF-7 and SKBR-3 cells. In addition, the fluorescence intensity seen in these experiments supported the notion that intrabody stability was increased by either 1) targeting expression of the sFv to the nucleus, thereby removing the protein from the harsh cytosolic environment (sFv vs sFv-NLS), or 2) addition of the Fc fragment, thereby increasing cytosolic (sFv vs sFv-Fc) as well as nuclear compartment (sFv-NLS vs sFv-Fc-NLS) stability.

Figure 3.



Key Research Accomplishments

- Completion of HE-12 and HE-172 single-chain antibody cloning
- Completion of HE-12 and HE-172 sFv-Fc mammalian expression vectors for use in expression, purification, and binding analysis of sFv-Fc proteins manufactured in a mammalian cell line
- Completion of HE-172 mammalian expression vectors for targeted cytosolic and nuclear targeting of sFv and sFv-Fc constructs
- We have shown sFvs can be expressed and successfully targeted to the cytosol and nucleus of the breast cancer cell lines MCF-7 and SKBR-3

Reportable Outcomes

There have been no publications, patents, licences, degrees earned, etc resulting from this funding as of this date. An abstract of this research was presented at the Era of Hope Breast Cancer Research Program Meeting in Atlanta, GA, June 8-12, 2000.

Conclusions

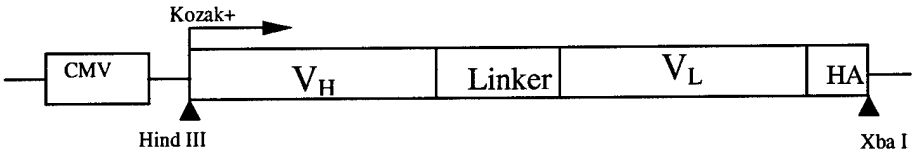
Intrabodies provide a powerful tool to examine the in vivo function of a given protein. We are using an intrabody approach to investigate the role aberrant cyclin E expression plays in breast cancer transformation and tumorigenesis by attempting to phenotypically knock out cyclin E. Toward this goal, we have generated two anti-cyclin E single chain antibodies, and displayed their ability to be expressed and targeted to the cytosol and nucleus of breast cancer cells. We are currently evaluating the binding affinity of the anti-cyclin E intrabodies, and will attempt to generate breast cancer cell lines stably or inducibly expressing these intrabodies. We will then evaluate the biological effects of intrabody expression on cyclin E activity in an attempt to elucidate the specific role cyclin E plays in the tumorigenicity and proliferation of breast cancer.

Appendix A. HE-12 and HE-172 sFv Nucleotide and Deduced Amino Acid Sequences

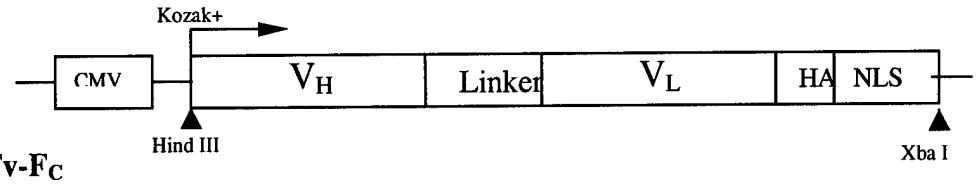
```
                          10         20         30         40         50
172SFV.SEQ          1 GAGGTGCAGC TGGAGGAGTC AGGACCTGAA CTGAAGAAGC CTGGAGAGAC        50
HE12SFV.SEQ         1 .......... ...T...... T........G .......... ..........        50
                          60         70         80         90        100
172SFV.SEQ         51 AGTCAGGATC TCCTGCAAGA CTTCTGGGTA TAACTTCACA ACTGCTGGAA       100
HE12SFV.SEQ        51 .....A.... .........G .......... ..C....... .AGTA.....       100
                         110        120        130        140        150
172SFV.SEQ        101 TGCAGTGGGT GCAAAAGATG CCAGGAAAGG GTTTGAAGTG GATTGGCTGG       150
HE12SFV.SEQ       101 ..A.C..... .A..C..GCT .........A ....A....T CT.G......       150
                         160        170        180        190        200
172SFV.SEQ        151 ATAAACACCC ACTCTGGAGT GCCAAAATAT GCAGAAGACT TCAAGGGACG       200
HE12SFV.SEQ       151 .........T ..A......A .....C.... ..T..T.... ..G.......       200
                         210        220        230        240        250
172SFV.SEQ        201 GTTTGCCTTC TCTTTGGAAA CCTCTGCCAG CACTGTATAT TTACAGATAA       250
HE12SFV.SEQ       201 ...G...... .......... .........A .....CC... ..G.....C.       250
                         260        270        280        290        300
172SFV.SEQ        251 ACAACCTCAA AGATGAGGAC ACGGCTACGT ATTTCTGTAC GAGAAATAAA       300
HE12SFV.SEQ       251 ...G...... .A........ .T......A. ........GT C------.C.T       300
                         310        320        330        340        350
172SFV.SEQ        301 TATAGGTACG AGGCCTGGTT TGCTTACTGG GGCCAAGGGA CTCTGGTCAC       350
HE12SFV.SEQ       301 ...---.... G--------- ----..T... .......... ..........       350
                         360        370        380        390        400
172SFV.SEQ        351 TGTCTCTGCA GGTGGCGGTG GCTCCGGAGG TGGTGGGAGC GGTGGCGGCG       400
HE12SFV.SEQ       351 .......... .......... .......... .......... ..........       400
                         410        420        430        440        450
172SFV.SEQ        401 GATCTGACAT TGTGCTCACA CAGTCGGCAG CAATCATGTC TGCATCTCCA       450
HE12SFV.SEQ       401 .......... ......G... ......C..C TC.CTT.... G.TGA.CATT       450
                         460        470        480        490        500
172SFV.SEQ        451 GGGGAGAGGG TCACCGTGTC CTGCA-GT-- -GCCAG---- -CTCAAGT--       500
HE12SFV.SEQ       451 ..AC.ACCA. C.T..A.C.. T....A..CA A.T...AGCC T..T.GA.AG       500
                         510        520        530        540        550
172SFV.SEQ        501 -----GTAAG TTACATTT-- ACTGGTACCA GCAGAAGCCA GGATCCTCCC       550
HE12SFV.SEQ       501 TGATG.CG.. AC.T....GA .T....TGTT A....G.... ..CCAG..T.       550
                         560        570        580        590        600
172SFV.SEQ        551 CCAAACCCTG GATTTATCGC ACATTCAACC TGGCTTCTGG AGTCCCTGCT       600
HE12SFV.SEQ       551 .A..G.G.CT A..C....TG GTG.CT..G. ...AC..... ........AC       600
                         610        620        630        640        650
172SFV.SEQ        601 CGCTTCAGTG GCAGTGGGTC TGGGACCTCT TACTCTCTCA CAATCAGCAG       650
HE12SFV.SEQ       601 A.G....C.. .......A.. A.....AGA. .TTA.A..G. A.........       650
                         660        670        680        690        700
172SFV.SEQ        651 CATGGAGGCA GAAGATGCTG CCACTTATTA CTGCCAGCAG TATCATA-GT       700
HE12SFV.SEQ       651 AG.......T ..G...TTG. GAGT...... T...TG...A -G.T.C.CA.       700
                         710        720        730        740        750
172SFV.SEQ        701 TATCCAGTCA CGTTCGGTGC TGGGACCAAG CTGGAGATCA AACGT.....       750
HE12SFV.SEQ       701 .T...TCAG. .........G A..C...... .......... ..........       750
```

```
                          10         20         30         40         50
HE12SFV.AMI         1 EVQLVESGPE LKKPGETVKI SCKASGYTFT KYGMNWVKQA PGKSLKFLGW        50
HE172SFV.AMI        1 ....E..... ........R. ...T...N.. TA..Q..QKM ...G..WI..        50
                          60         70         80         90        100
HE12SFV.AMI        51 INTYTGEPTY ADDFEGRLAF SLETSANTAY LQINSLKNED MATYFCV---       100
HE172SFV.AMI       51 ...HS.V.K. .E..K..F.. ......S.V. ....N..D.. T.....TRNK       100
                         110        120        130        140        150
HE12SFV.AMI       101 ----SLLRYW GQGTLVTVSA GGGGSGGGGS GGGGSDIVLT QSPLTLSVTI       150
HE172SFV.AMI      101 YRYEAWFA.. .......... .......... .......... ..AAIM.ASP       150
                         160        170        180        190        200
HE12SFV.AMI       151 GQPASISCKS SQSLLDSDGE TYLNWLLQRP GQSPKRLIYL VSKLDSGVPD       200
HE172SFV.AMI      151 .ERVTV..SA .S.V------ S.IY.YQ.K. .S...PW..R TFN.A....A       200
                         210        220        230        240        250
HE12SFV.AMI       201 RFTGSGSGTD FTLKISRVEA EDLGVYYCWQ VTHFPQTFGG GTKLEIKR..       250
HE172SFV.AMI      201 ..S......S YS.T..SM.. ..AAT...Q. YHSY.V...A ..........       250
```

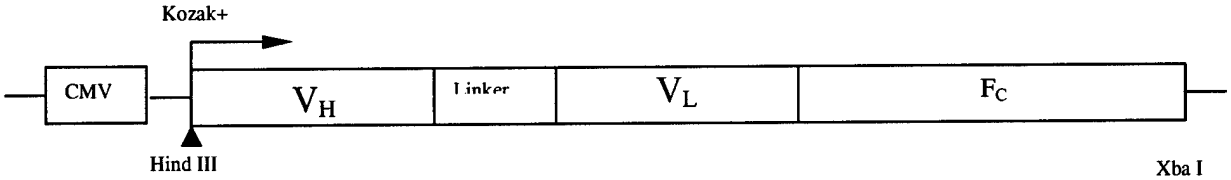
Appendix B. Schematic Representation ofAnti-cyclin E sFv and sFv-Fc Constructs.



sFv-HA-NLS



sFv-F_C



sFv-F_C-NLS